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Purification of an endoproteinase that digests the wheat 'Em' protein in vitro, and determination of its cleavage sites

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Germinating wheat embryos contain two endoproteolytic activities which digest the prominent 'Em' polypeptide These are easily assayed in clarified embryonic homogenates and are distinguishable by the pattern of their peptide products and by their different pH optima. One activity has a pH optimum of 4.0; the second activity is a cysteine endoproteinase with a preference for the 'Em' protein as its substrate. It is maximally active between pH 5.5 and 6 at 25°C. Analysis of the early cleavage products of the cysteine proteinase indicates scissile bonds between residues Glu^{32}-Ala^{33} and Asn^{36}-Leu^{37} in the 'Em' polypeptide. This endoproteinase has been purified and identified as a single polypeptide species of ca. 38,000 kDa.

Em polypeptide; Cysteine proteinase, Wheat embryo; Germination

1. INTRODUCTION

The course of embryonic development in cereals is marked by dramatic changes in the pattern of metabolic activity. During embryogenesis the course of metabolism is essentially anabolic, leading to the accumulation of storage reserves and of compounds associated with anhydrobiosis [1–3]. The embryogenic programme is closed by a progressive dehydration which results in the cessation of metabolic activity and the formation of a quiescent embryo. Upon subsequent uptake of water, a catabolic metabolic programme is initiated in which the stored reserves are mobilised to fuel the explosive germinative growth of the embryo, and in which compounds related to desiccation tolerance are degraded [4,5].

Conspicuous among the gene products which accumulate during wheat embryogenesis is the 'early-methionine-labelled' ('Em') protein [6,7]. This is a low M_r (ca. 9,900) polypeptide believed to play a part in mediating the acquisition of desiccation tolerance [8]. The 'Em' protein comprises the single most abundant soluble polypeptide present in quiescent (dry) embryos. Upon imbibition of water, the Em protein is rapidly and selectively degraded, completely disappearing from the polypeptide population within 24 hours [6,9].

In analysing the mechanism by which the 'Em' protein is degraded during germination, we have identified a cysteine endoproteinase activity from imbibing embryos which displays a remarkable selectivity towards the 'Em' polypeptide, in vitro [9]. Here we determine the optimal conditions of pH and temperature for its activity. Additionally, we have identified the principal cleavage sites within the 'Em' polypeptide and purified the enzyme to homogeneity.

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2. MATERIALS AND METHODS

2.1. *Plant material*

Clarified homogenates were prepared from 16-h germinated wheat embryos as previously described for the analysis and purification of proteinase activity directed against the wheat 'Em' protein [9]; this activity was identified by electrophoretic resolution of radiolabelled 'Em'-derived peptides following digestion of radiochemically pure 'Em' polypeptide, synthesised in vitro

2.2. *Optimisation of activity*

Conditions for optimal digestion of the 'Em' polypeptide were established using clarified extracts (12,000 × *g* supernatants) from germinating wheat embryos Equal quantities of radiochemically pure ^{35}S-labelled 'Em' polypeptide was incubated with extracts for up to 6 h under conditions of varying temperature and pH. For the pH range 3.0–6.0, reaction mixtures were buffered with 25 mM sodium citrate buffer. For the pH range 5.2–8.5, reaction mixtures were buffered with 25 mM Tris-maleate buffer. Following digestion, the reaction products were resolved by dodecylsulphate polyacrylamide gel electrophoresis [10] and detected by fluorography of the dried gels after impregnation with Amplify (Amersham, UK). Proteinase activity was estimated by determining the proportion of 'Em'-derived radioactivity remaining in the native polypeptide relative to that in the native polypeptide plus derived peptides. These values were obtained for each track by scanning densitometry of the X-ray film using an LKB 'Ultroscan' densitometer and integrating the peak areas. The values cited in figures 1b and 2b are expressed as relative absorbance values.

2.3. *Purification of 'Em'-endoproteinase*

'Em'-specific proteinase activity was purified from homogenates by sequential ammonium sulphate precipitation, organomercurial affinity chromatography, gel filtration and ion exchange chromatography: The fraction of proteins precipitating between 40% and 65% $(NH_4)_2SO_4$ was recovered by centrifugation and redissolved in 25 mM sodium citrate-phosphate buffer pH 5 5, containing 0.02% sodium

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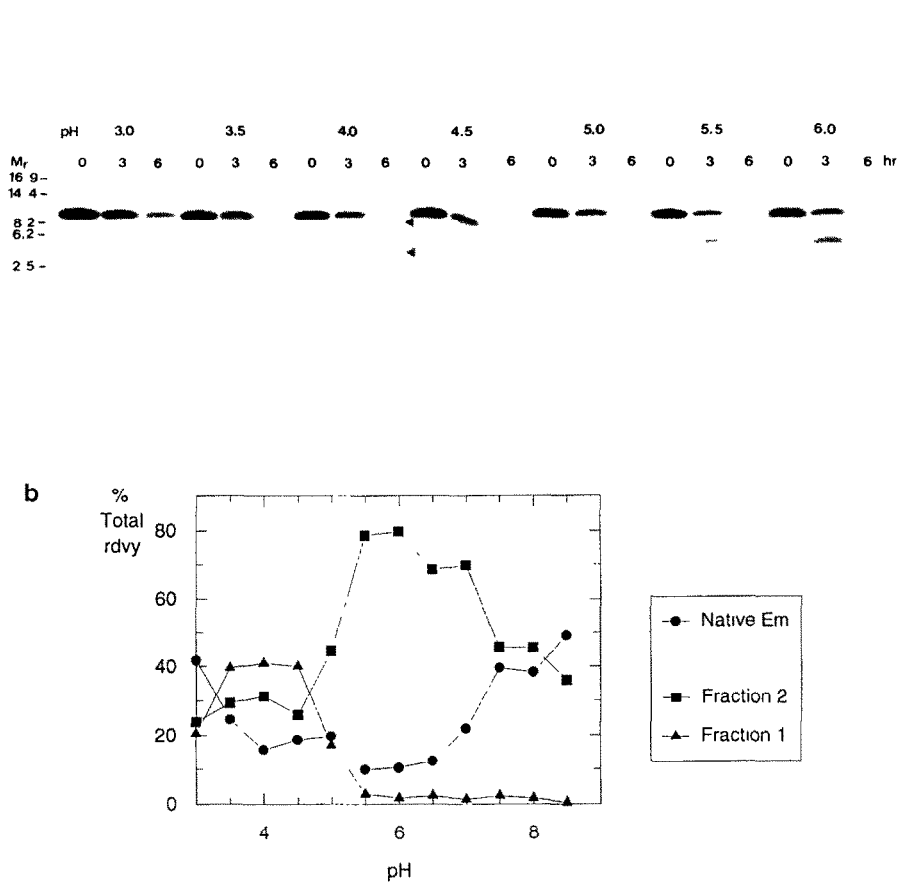

Fig. 1. pH dependence of proteolysis. (a) ^{35}S-labelled 'Em' polypeptide was incubated with clarified homogenates of germinating wheat embryos for 0, 3 and 6 h as indicated. The products were resolved by dodecylsulphate polyacrylamide gel electrophoresis and detected by fluorography. The migration of molecular mass standards is indicated ($M_r \cdot 10^{-3}$) and the peptides derived from the 'Em' protein by the action of the 'Fraction 1' protease are marked with arrowheads. (b) The fraction of radioactivity associated with the native 'Em' polypeptide and with the peptides derived either by 'Fraction 2' activity was determined by scanning densitometry of the electrophoretically resolved products of the 6-h digestion at each pH, in the fluorograph in Fig. 1a

azide. This was desalted by passage through a Sephadex G-25 column, and the eluate applied to an organomercurial agarose column (Affigel 501; Bio-Rad) equilibrated with 25 mM sodium citrate-phosphate buffer. Fractions eluting with column buffer containing 10mM dithiothreitol were assayed for 'Em'-proteinase activity and concentrated by addition of $(NH_4)_2SO_4$ to 95% saturation and centrifugation. The pellet was dissolved in 25 mM citrate-phosphate buffer pH 5.5, 25 mM NaCl, 1 mM dithiothreitol, 0.02% NaN_3 and applied to a Sephadex G-100 column. Proteinase-containing fractions were finally loaded on a DEAE-cellulose column and eluted with a continuous gradient (0.05–2 M) of NaCl.

2.4. *Identification of cleavage sites*

Pure 'Em' polypeptide was isolated from commercial wheat-germ by a modification of the procedure of Grzelczak et al. [6]. Purification of the 'Em' polypeptide was greatly enhanced if clarified homogenates (30,000 × *g* supernatants) were heated at 100°C for 10 min prior to fractionation. Whereas the bulk of the proteins present in the homogenate are denatured and rendered insoluble by this procedure, a small subfraction, including the 'Em' polypeptide, are boiling-stable and remain in the supernatant after subsequent recentrifugation (10,000 × *g*, 20 min). Pure 'Em' polypeptide was incubated with 'Em'-protease extract (6 h, 22°C, pH 5.5) and subjected to electrophoretic separation. The resulting peptides were transferred by electroblotting to 'Fluorotrans' membrane (Pall Europe Ltd., Portsmouth, UK) for determination of N-terminal amino acid sequences using an Applied Biosystems 477A liquid-pulse sequencer. Sequence determination was performed in the SERC Protein Sequence facility at Leeds.

3. RESULTS AND DISCUSSION

3.1. *Optimisation of activities*

The 'Em' polypeptide is digested in vitro by endoproteinases extracted from germinating wheat embryos. Using clarified wheat embryo homogenates we have defined the optimal conditions for these activities. The pH optimum for 'Em' proteolysis was determined over the range pH 3 to pH 8.5. In preliminary experiments, proteolysis was found to be relatively reduced above pH 6.5. Fig. 1 illustrates the pattern of 'Em' digestion by extracts across the pH range 3.0–6.0, within which range two endoproteolytic activities could be distinguished. We have previously identified these activities as a minor, non-selective component and a major component which exhibits a strong selectivity towards the 'Em' protein. These two activities are separable by

DEAE-cellulose chromatography into two fractions ('Fraction 1' and 'Fraction 2', respectively). We have previously shown the major, Em-specific, 'Fraction 2' component to be a cysteine endoproteinase [9].

In unfractionated homogenates these activities are also distinguishable by the different patterns of peptides derived from the 'Em' polypeptide. The 'Fraction 1' activity yields the two peptides marked by arrowheads in Fig. 1a, the 'Fraction 2' activity yields the remaining peptides [9]. It is evident from Fig. 1 that these two activities differ in their pH optima. The 'Fraction 1' activity is maximally active at pH 4.0, whereas the 'Fraction 2' activity is maximal at pH 5.5–6, a pH at which the 'Fraction 1' activity is virtually undetactable. The relative contributions of these activities to 'Em' proteolysis can be estimated by determining the quantity of radioactivity associated with the 'Fraction 1'-derived peptides and the 'Fraction 2'-derived peptides, respectively (Fig. 1b).

Previous studies of protease activities within germinating wheat embryos have utilised assays based on the digestion of protein substrates of heterologous origin (e.g. casein) [11,12]. These assays have thus, necessarily, only sufficed to identify activities with a broad substrate specificity. These activities have been shown to be enzymes with low pH optima (ca. pH 4), and the 'Fraction 1' activity appears to be just such an enzyme. By contrast, the 'Em'-specific activity, with a pH optimum between 5.5 and 6, is clearly distinguishable from these non-selective activities.

The activity of the 'Fraction 2' endoproteinase was assayed over a range of temperatures, by incubation of radiolabelled 'Em' polypeptide with extracts at pH 5.5 (thereby eliminating 'Fraction 1' activity). The results, illustrated in Fig. 2, demonstrate that this activity yields only the 'Fraction 2'-specific peptides, their radioactivity being in inverse proportion to that remaining in the native 'Em' band. No 'background' of heterodisperse material, which would be diagnostic of contaminating exoprotease, was observed. The 'Fraction 2' activity was maximal between 25°C and 30°C, but declined at higher temperatures, a range of temperatures unsurprising for an enzyme of plant origin, being in accordance with the optimal temperatures seen for the bulk of wheat embryo enzyme activities, in vitro (e.g. cell-free translation), which similarly decline above 30°C.

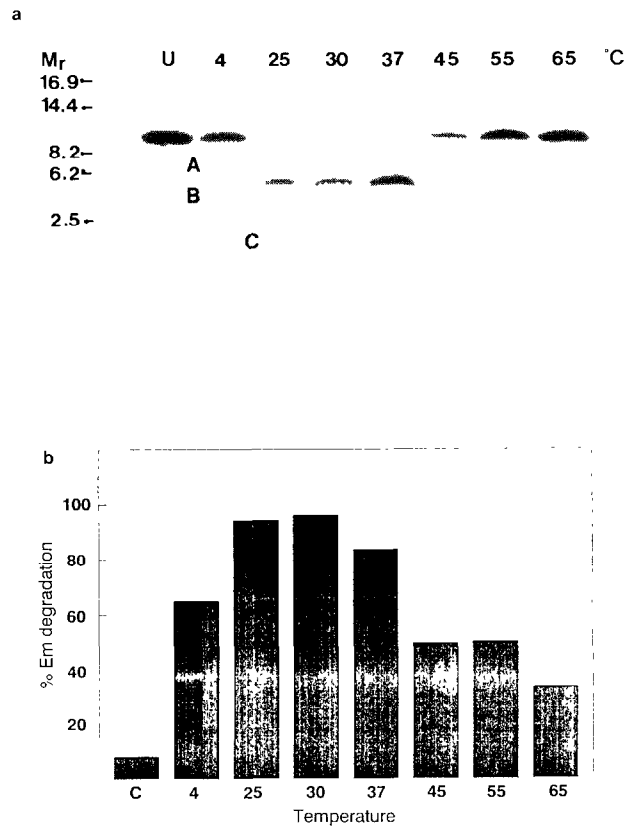

Fig. 2. Temperature dependence of proteolysis. (a) 'Fraction 2' activity was determined at a range of temperatures (as indicated; U = undigested) by incubating ^{35}S-labelled 'Em' polypeptide with a clarified homogenate of germinating wheat embryos for 6 h at pH 5.5. The products were resolved by dodecylsulphate polyacrylamide gel electrophoresis and detected by fluorography. The migration of molecular mass standards ($M_r \cdot 10^{-1}$) is indicated. (b) Relative activity of the 'Fraction 2' protease at different temperatures. Since the decline in radioactivity of the 'Em' band was accompanied specifically by a concomitant increase in the 'Fraction 2'-specific peptides, the '% Em degradation', determined by scanning densitometry of the fluorograph in Fig. 2a (corresponding to 100−(% total radioactivity in native 'Em') for each track) directly represents the activity of this protease.

3.2. *Cleavage specificity*

Because the 'Fraction 2' endoproteolytic activity is highly substrate-specific, preferentially digesting the 'Em' polypeptide [9], the identification of the cleavage sites within this protein is clearly of interest. Analysis of the time-course of 'Em' digestion by the 'Fraction 2' activity have indicated that the first products to appear are the peptides of M_r ca. 6,500 and 5,000 (designated 'A' and 'B' in Fig. 2a) followed by a peptide of M_r ca. 2,000 ('C') [9]. Bulk quantities of these peptides were obtained by digestion of purified 'Em' polypeptide with the 'Fraction 2' proteinase and were isolated by electroblotting from polyacrylamide gels for N-terminal amino acid sequence determination. Inspection of the N-terminal sequences of peptides 'A' and 'B' revealed that their N-terminal residues respectively corresponded to Ala^{33} and Leu^{37} in the 'Em' polypeptide sequence [13,14] (Fig. 3).

The early appearance of the 'A' and 'B' peptides is strongly indicative of their being the initial cleavage products produced by the 'Em'-protease. The sequence determination of these peptides thus identifies the presumptive scissile bonds first recognised by the endopeptidase as glu-ala and asn-leu. Thiol proteases implicated in the mobilisation of storage globulins in legume seeds have been identified as exhibiting cleavage adjacent to

1	met	ala	ser	gly	gln	gln	glu	arg	ser	gln	leu	asp	arg	lys	ala
16	arg	glu	gly	glu	thr	val	val	pro	gly	gly	thr	gly	gly	lys	ser
31	leu	glu	*ala	gln	glu	asn	*leu	ala	glu	gly	arg	ser	arg	gly	gly
46	gln	thr	arg	arg	gln	glu	met	gly	glu	glu	gly	tyr	ser	glu	met
61	gly	arg	lys	gly	gly	leu	ser	thr	asn	asp	gln	ser	gly	gly	glu
76	arg	ala	ala	arg	gln	gly	ile	asp	ile	asp	glu	ser	lys	phe	lys
91	thr	lys	ser	TER.											

Fig. 3. Cleavage sites within the 'Em' polypeptide. The N-terminal sequences of peptides 'A' (underlined) and 'B' (overlined) were determined and compared with the amino acid sequence of the 'Em' polypeptide derived from the cDNA clone (pMTK1a) from which the radiolabelled 'Em' substrate was prepared. The presumptive cleavage sites are denoted by '*'.

asparagine residues [15] as has the endopeptidase responsible for the processing of proglobulins within protein storage vacuoles during seed development [16,17]. However, it will clearly be necessary, in future studies, to determine the contribution made by surrounding residues to the enzyme's cleavage specificity.

The 'C' peptide, which appears after longer periods of digestion, with a concomitant decline in the recovery of the larger peptides, has been suggested to be a secondary product of the continued digestion of these primary products [9]. N-terminal analysis of the 'C' peptide proved unrevealing, as this component apparently comprised a mixture of cleavage products which could not satisfactorily be resolved.

3.3. *Purification*

The 'Em-endoproteinase' was purified by sequential fractionation procedures, with eluates from the various columns being assayed for their ability to generate the characteristic 'Fraction 2'-specific pattern of peptides from the radiolabelled 'Em' protein. The strategy for the purification of the 'Em-endoproteinase' was informed by the discovery that this enzyme was a cysteine endoproteinase. Thus, following a preliminary fractionation by ammonium sulphate precipitation, a fractionation by affinity chromatography on organomercurial agarose was performed [18]. This step resulted in a substantial purification of the activity, the fraction containing only a small number of polypeptide species when analysed by dodecylsulphate polyacrylamide gel electrophoresis (Fig. 4). The principal component in this fraction was a polypeptide of M_r ca. 38,000. This polypeptide was also recovered following the subsequent steps in the purification protocol. Following the final, ion-exchange chromatography, step this polypeptide was the only remaining component found in the active fraction.

Three properties of this enzyme: its relative molecular mass, its mode of cleavage (as a cysteine proteinase) and its activity during germination raise the possibility that it may be a cathepsin-like proteinase [19]. A number of cathepsin-like proteinases have recently been identified in germinating cereal grains, where they are thought principally to be involved in the mobilisation of endosperm storage reserves [19–21]. Whilst most of these enzymes are principally synthesised in and secreted from the aleurone layer, there is evidence to suggest that a gibberellic acid-regulated gene encoding a cathepsin-B homologue is expressed within the scutellum of germinating wheat embryos [22]. The purification of this enzyme allows us to investigate this possibility, and through the development of molecular probes, will permit the determination of the means by which the synthesis and activity of the 'Em-protease' is regulated during embryogenesis and germination.

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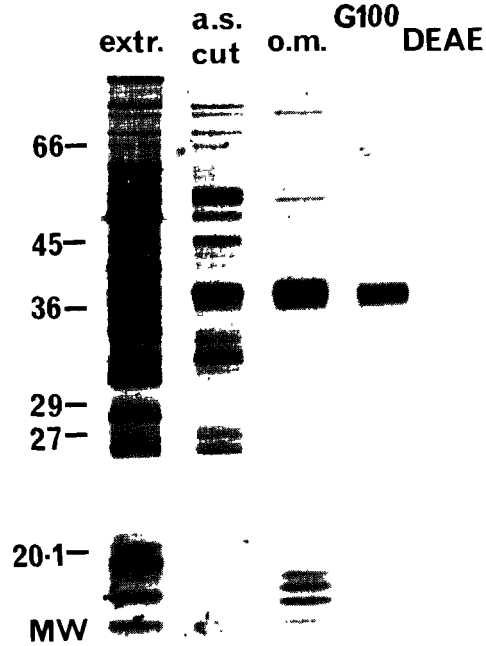

Fig. 4. Purification of the 'Em'-proteolytic activity recovered at each stage of the purification protocol were analysed by dodecylsulphate polyacrylamide gel electrophoresis. Polypeptides were revealed by silver-staining. The fractions comprise: 'extr.': total protein in the clarified homogenate (6 μg). 'a.s. cut': proteins precipitating between 40% and 65% $(NH_4)_2SO_4$ (6 μg). 'o.m.': fractions recovered from organomercurial agarose (6 μg). 'G-100': fractions recovered after filtration through Sephadex G-100 (1 μg). 'DEAE': fractions recovered after ion-exchange chromatography on DEAE-cellulose (ca. 50 ng). The migration of molecular mass standards ($M_r \cdot 10^{-3}$) is indicated.

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